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14. ABSTRACT

Stathmin is a microtubule destabilizing protein that interacts with two tubulin heterodimers to form a ternary stathmin-tubulin complex. This interaction is critical for the dynamic regulation of interphase & mitotic microtubules. Here, we asked whether intracellular delivery of stathmin-like fusion peptide(s) could prevent the normal association of stathmin with tubulin and mediate an anti-proliferative effect in *in vitro* models of human breast cancer. Evaluation of intracellular uptake of the peptide(s) demonstrated that breast cancer cells could effectively take up the designed peptides. Proliferation assays showed significant growth inhibitory effects in breast cancer cells exposed to wild type or mutant peptides compared to cells exposed to a control peptide. Furthermore, immunofluorescence analysis of breast cancer cells exposed to the designed peptides showed marked inhibition of microtubule polymerization. This suggests that the observed anti-proliferative effect is likely a result of inhibition of microtubule assembly. Our current studies are aimed towards evaluating the biologic effects of the fusion peptide(s) in the absence & presence of vinblastine to determine if the fusion peptide(s) interact synergistically with vinblastine in breast cancer cells. These studies should provide the proof-of-principle that peptide(s) could be used to modulate stathmin function and inhibit malignant proliferation of breast cancer cells.

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A. INTRODUCTION

Breast cancer is one of the most frequent malignancies and is the major leading cause of cancer-related deaths in women in the U.S. When local therapies for breast cancer fail and the disease progresses, systemic estrogen ablation therapy, with or without chemotherapy, can lead to tumor regression. However, the disease inevitably progresses to an estrogen-independent state that becomes resistant to hormonal therapy and chemotherapy. In this advanced stage, there are currently no curative therapies as such patients almost always die from their metastatic disease. Thus, there is clearly a pressing need for the development of alternative strategies for this devastating malignancy.

Stathmin is the founding member of a family of microtubule destabilizing proteins that regulate the polymerization & depolymerization of the microtubules through its cell cycle specific phosphorylation [1-4]. Numerous studies, including our own, first demonstrated that stathmin plays an important role in the regulation of cell proliferation [2, 4-6]. This was followed by the identification of stathmin as a major regulator of the dynamics of microtubules that make up the mitotic spindle [7]. Thus, stathmin is one of the key regulators of the microtubule cytoskeleton and the mitotic spindle [1, 2, 4, 7, 8]. Stathmin promotes microtubule depolymerization either by increasing the rate of microtubule catastrophe or by sequestering tubulin thus depleting the pool of tubulin available for polymerization [1, 7, 9-11]. Both activities of stathmin are modulated by the interaction of stathmin to two tubulin heterodimers to form a ternary (T2S) stathmin-tubulin complex that directly contributes to the dynamic regulation of microtubules during cell cycle progression [7, 9, 10, 12]. In addition to its well-documented role in cellular proliferation, stathmin is also expressed at high levels in a wide variety of human malignancies including breast cancer. Of particular significance, the high level of stathmin expression has been shown to correlate with the malignant behavior of breast cancer cells, proliferation cell nuclear antigen (PCNA) expression, large tumor size, high tumor stage and poor prognosis [13-15]. Thus, the level of stathmin expression serves as an important prognostic marker that predicts survival in breast cancer and provides an attractive target for breast cancer therapy.

Recently, numerous short peptides from the N-terminal regions of different members of stathmin family were shown to impede tubulin polymerization with different efficiencies [16]. The inhibition of tubulin polymerization was a result of direct stoichiometric interaction of the peptide with tubulin to form a peptide/tubulin complex [16]. The most efficient of these peptides, I19L, was a 19-residue peptide that covers the N-terminal domain of stathmin (residue 6-24) [16]. The ability of I19L to impede tubulin polymerization was also found to be markedly reduced by Ser-16 phosphorylation [16]. Thus, this peptide possesses an autonomous anti-MT activity [16]. A major goal of our study is to test the hypothesis whether intracellular delivery of stathmin-like peptide(s) would interfere with the normal association of stathmin with tubulin and inhibit the malignant proliferation of breast cancer cells by disrupting microtubule assembly and the spindle apparatus. Unlike taxanes that stabilize microtubules, vinca alkaloids destabilize microtubules. The combination of stathmin-based peptide(s) with vinca alkaloids is particularly attractive since both agents inhibit microtubule assembly. Thus, a secondary hypothesis is whether stathmin-like peptide(s) would interact synergistically with vinblastine in breast cancer cells *in vitro*.

B. BODY

In our grant application entitled "Novel antimicrotubule agents for breast cancer", we had proposed three specific aims: 1) To test the ability of stathmin-like peptide(s) to bind tubulin and impede

microtubule assembly in *in vitro* models of breast cancer cell lines; 2) To test the biologic effects of the peptides on proliferation, clonogenicity & apoptosis in different breast cancer cell lines *in vitro*; & 3) To determine if the combination of stathmin-like peptide(s) & anti-microtubule drugs like vinblastine would result in synergistic anti-tumor effects in breast cancer cell lines. In this section, we will briefly summarize the experiments that have been done so far to achieve the above specific goals. It should be noted that this report does not represent the final report. Due to medical reasons, the proposed studies could not be completed by the end of the grant term. Therefore, the PI had requested a no cost extension to complete all the proposed studies.

1. DESIGN OF THE STATHMIN-LIKE PEPTIDES:

The peptide(s) that we designed consisted of the same 19 residues (residues 6-24) from the N-terminal domain of stathmin [16]. This peptide was fused to a TAT transduction domain (residues 48-60) [17], that served as a carrier to facilitate entry into breast cancer cells. In order to be able to track this fusion peptide intracellularly, we also included a hemagglutinin (HA) epitope tag. This peptide was named wild-type stathmin peptide (W-SP). Since W-SP could be phosphorylated & inactivated intracellularly by $p34^{cdc2}$ kinase [5, 16], we also made another peptide in which Ser-16 was mutated to an alanine to prevent its inactivation by phosphorylation. Previous studies had shown that this substitution results in a more potent form of stathmin that cannot be inactivated by phosphorylation [16]. The resulting mutant peptide was named W-SaP. As a control, we generated a similar peptide in which the TAT and HA sequences were identical to W-SP but the stathmin-like domain was totally scrambled (Sc-P). All peptides were chemically synthesized at >95% purity at GenWay Biotech, Inc.

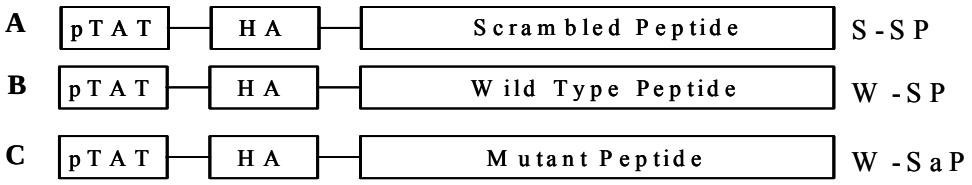

Fig.1 Schematic illustration of different fusion peptides. The domains are labeled. A. Design of scrambled peptide (Sc-P). B. Design of wild-type stathmin peptide (W-SP). C. Design of mutant stathmin peptide (W-SaP). The single difference between the two peptides, W-SP & W-SaP, is the substitution of the serine-16 to alanine to prevent in activation of the peptide by phosphorylation.

2. INTRACELLULAR TRAFFICKING OF THE DESIGNED PEPTIDES:

To determine whether the designed peptide(s) are taken up intracellularly, we first tracked the intracellular uptake of the peptide(s) in breast cancer cells exposed to the different peptide(s) by immunostaining with Alexa Fluor 488 conjugated anti-HA antibody. Since stathmin's role in mitosis starts by promoting the depolymerization of interphase microtubules before the breakdown of the nuclear membrane [1, 2], we had used a mutant TAT sequence that was previously shown to maintain the cargo peptide in the cytoplasm [18]. Fig. 2 shows representative images of T47D breast cancer cells exposed to different peptides. The presence of the peptide(s) was detected primarily in the cytoplasm of breast cancer cells as expected (Fig.2). Furthermore, nearly 85% **of**

the breast cancer cells were positiv e for HA staini ng suggesting that a vast m ajority of cells had taken up the peptide(s). Sim ilar findings were observed in othe r b reast cancer cells includ ing SKBR-2 and MCF-7 breast cancer cell lines.

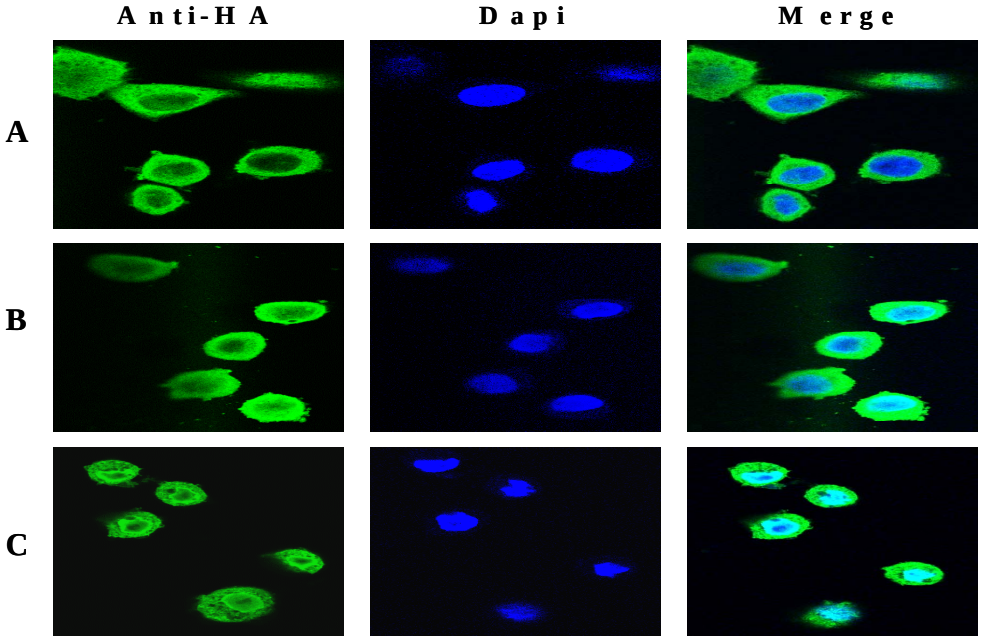

Fig.2 Intracellular localization of the designed peptide(s) by confocal microscopy. These are representative im ages of T47D breast cancer cells that were exposed to different peptide(s) overnight. Next day, the cells were fixed, permeabilized and stained with Alexa Fluor 488 conjugated anti-HA antibody. A, B & C are represen tative images of T47D cells exposed to Sc-P, W-SP and W -SaP peptides respectively. The left panel shows im ages stained with Alexa Fluor 488 conjugated anti-HA antibody, the middle panel shows images stained with DAPI and the right panel shows images in which the Alexa and DAPI staining were merged.

3. EFFECTS OF THE DESIGNED PEPTIDES ON THE PROLIFERATION OF BREAST CANCER CELLS:

Since this peptide m imics the tubulin sequesteri ng activity of the entire stathm in molecule, w e predicted that its pres ence in cancer cells would prom ote microtubule depolym erization as w as previously observed in stathm in over-expressing cells [19]. This in turn should prevent the formation of a functional m itotic spindle and mediate an anti-proliferative effect. Thus, we examined the effects of the designed peptides on the rate o f prolif eration of breast cancer cells using a non-radioactive MTT (Met hylthiazoletetrazolium) assay. This assay is based on the conversion of tetrazolium salt into a blue form azon dye [20]. Growth curves of breast cancer cell lines exposed to different peptides are shown in Fig.3. W hen breast cancer cells were exposed to control Sc-P at different concentrations, the rate of proliferation was essentially unchanged and the cells continued to proliferate lik e the untreated cells (Fig.3, left panel). Exposure of breast cancer cells to wild -type stathmin peptide, W-SP, at a lower concentration of 2 uM showed a m oderate decrease in the rate of proliferation in the different cell lines (Fig.3, middle panel). However, when the concentration of the W -SP was increased to 5 uM, the rate of proliferation was markedly

decreased in all three cell lines as shown in Fig.3 (middle panel). Interestingly, when breast cancer cells were exposed to the mutant peptide, W-SaP, at the same concentrations (2 and 5 uM), the growth inhibitory effects were much more pronounced even at a lower concentration of 2 uM and there was a complete cessation of growth at a concentration of 5 uM (Fig.3, right panel). In other words, exposure of breast cancer cells to low concentrations (2 uM) of wild-type stathmin peptide moderately inhibited proliferation, while exposure to the same concentration of mutant peptide was sufficient to result in a near complete suppression of growth. Thus, this data demonstrates that both, wild-type & mutant peptides can inhibit the growth of breast cancer cells *in vitro*. However, the mutant peptide mediates a more profound anti-proliferative effect. This is not surprising since the mutant peptide cannot be inactivated by phosphorylation as discussed above.

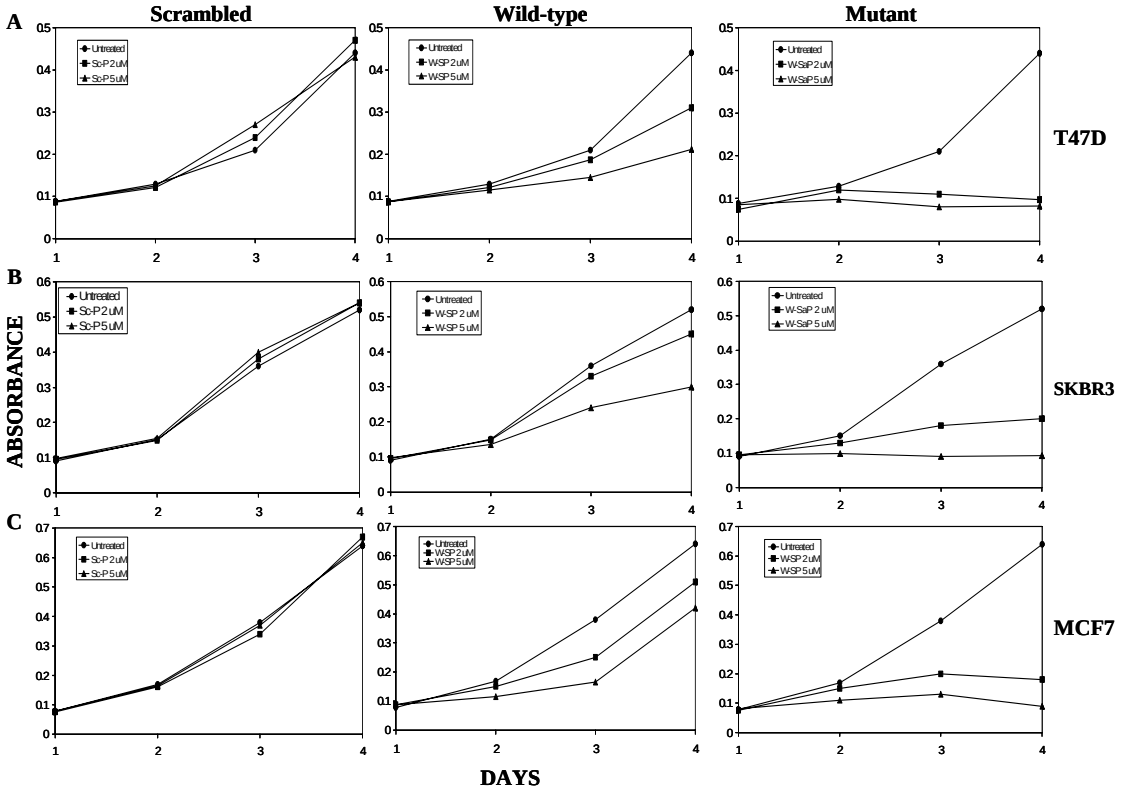

Fig.3 Effects of the designed peptides on the rate of proliferation of breast cancer cell lines. Breast cancer cells were plated equally at a density of 1000 cells/well in 96 well plates and exposed to either Sc-P, W-SP or W-SaP in triplicates overnight. The media was replaced with fresh medium on the next day & the cells were incubated at 37° C for 4 days. The cells were stained with MTT for 3 hours and absorbance was measured at 570 nm in a microplate reader. A. Growth rates of untreated T47D cells or T47D cells exposed to Sc-P, W-SP or W-SaP peptide at different concentrations as indicated. B. Growth rates of untreated SKBR3 cells or SKBR3 cells exposed to Sc-P, W-SP or W-SaP peptide at different concentrations as indicated. C. Growth rates of untreated MCF7 cells or MCF7 cells exposed to Sc-P, W-SP or W-SaP peptide at different concentrations as

indicated. The growth curves were generated by plotting the means of triplicate absorbance measurements based on cellular conversion of tetrazolium salt.

4. EVALUATION OF MICROTUBULE ORGANIZATION:

Stathmin is one of the key regulators of the microtubule cytoskeleton that regulate the dynamics of microtubules by promoting microtubule depolymerization. Since the N-terminal stathmin peptide was previously shown to impede microtubule assembly in *in vitro* polymerization assays [21], we asked whether intracellular delivery of the designed peptides would result in destabilization of microtubules in breast cancer cells. Thus, we examined the effects of different peptides on the microtubule cytoskeleton in breast cancer cells by immunofluorescence analysis of microtubules (Fig. 4) as we had previously described [4, 22]. Exposure of breast cancer cells to control Sc-P showed normal organization of microtubule network (Fig.4A). In contrast, when cells were exposed to W-SP or W-SaP, there was a reduction in microtubule density (Fig.4B & C) compared to control cells (Fig.4A). However, the reduction in microtubule density was much greater in cells exposed to mutant W-SaP (Fig.4C) than in cells exposed to wild type W-SP (Fig.4B) as expected. A similar decrease in microtubule density was also observed in SKBR3 and MCF7 breast cancer cells. This data demonstrates that the designed peptides can inhibit microtubule polymerization in a cellular environment. Thus, the pronounced anti-proliferative effect observed with mutant peptide is likely a result of inhibition of microtubule assembly.

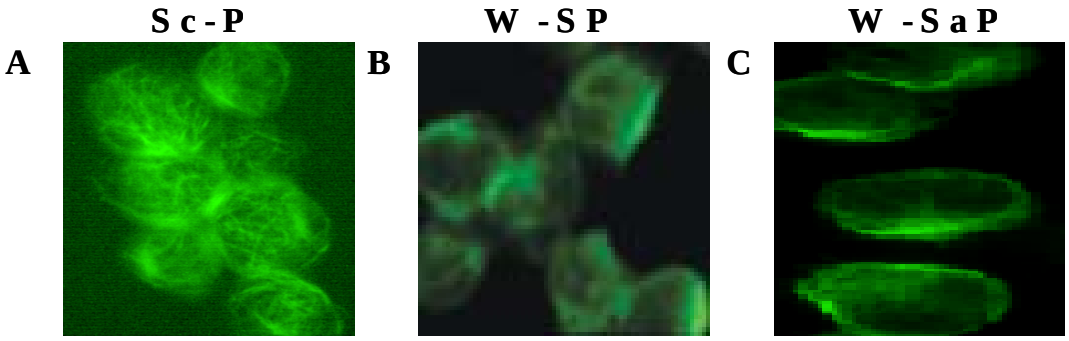

Fig.4 Effects of the designed peptides on the microtubule cytoskeleton of breast cancer cells. These are representative images of T47D cells exposed to different peptides. Cells plated in chamber slides were exposed to either Sc-P, W-SP or W-SaP overnight. Next day, the cells were fixed & stained with fluorescein conjugated anti-tubulin antibody. The images were captured at 100x under oil immersion. A, B & C are representative photograph of MT network in cells treated with Sc-P, W-SP & W-SaP respectively.

C. KEY RESEARCH ACCOMPLISHMENTS

(i) The experiments described above demonstrate that breast cancer cells can effectively take up the designed stathmin-like peptides.
(ii) We also demonstrate that intracellular delivery of stathmin-like peptides can markedly inhibit the proliferation of breast cancer cells *in vitro*.
(iii) We also demonstrate that the same peptides can interfere with the assembly of microtubules.

D. REPORTABLE OUTCOMES

The research summarized in this report is being planned to be disseminated at the Era of Hope Breast Cancer meeting. Towards the end of the no cost extension period, we aim to complete all the studies that we had proposed in the grant application. We will also plan to submit another abstract to the American Association of Cancer Research meeting. This abstract will be focused on the effects of stathmin-like peptides in combination with anti-microtubule drugs like vinblastine in breast cancer cells *in vitro.*

E. CONCLUSIONS

Based on the studies completed so far, we conclude that the designed stathmin-like peptides are capable of inhibiting the growth of breast cancer cells *in vitro.* The observed anti-proliferative effects are likely a result of inhibition of microtubule assembly.

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